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OVER-EXPRESSION OF *ARABIDOPSIS PHYTOCHELATIN SYNTHASE* GENE IN *ORYZA SATIVA* CONFERS ENHANCED TOLERANCE TO HEAVY METAL CADMIUM

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ABSTRACT

The heavy metal cadmium (Cd) is one of the most toxic elements. It decreases the growth rate of plants by affecting various aspects of metabolism mainly in normal uptake and utilization of macro and micronutrients. To overcome these challenges Phytochelatingenes were over expressed and developed Cd tolerance in different plants. Phytochelatins, a class of post translationally synthesized peptides, play a key role in Cd tolerance in plants by chelating the Cd substances and decreasing their free concentrations. This investigation mainly deals with cloning of Arabidopsis phytochelatingene (AtPCS1) and its effects on tolerance to Cd in transgenic rice plants. We have constructed a plant expression pCambia 1302-hyg-CaMV35S-AtPCS1-polyA-GFP binary vector, and transferred into rice calli employing Agrobacterium-mediated genetic transformation method. The transgenic plants were regenerated and grown to maturity in the glasshouse. Subsequently, PCR, Southern blot and Northern blot analyses was carried out to confirm the presence, copy number and expression of AtPCS1 gene in transgenic rice plants, respectively. Later, the homozygous lines were identified by germinating T_2 resistant seeds on selection medium containing hygromycin (50 mg/L). Two homozygous transgenic rice lines ($SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$) expressing AtPCS1 of T_3 generation along with un-transformed controls were used for further Cd tolerance analyses. Effect of Cd on root and shoot growths of AtPCS1 rice transformants and Untransformed Control (UC) were measured. Likewise, the accumulation of Cd in roots and shoots of AtPCS1 transgenic rice lines along with untransformed controls were also measured. The transgenic rice lines showed marked enhancement in tolerance to Cd and also disclosed increased accumulation of Cd in roots and shoots

KEYWORDS: Phytochelatins, Phytochelatin Synthase & Transgenic Rice

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Original Article

1. INTRODUCTION

Environmental contamination with heavy metals is implicated in many plants and causes crop losses. This universal problem poses a challenge with a price tag for remediation, estimated at upward of $200 billion in the US alone (Abhilash et al. 2009). In Bioremediation, microbes and plants are used as ecological remediators to reclaim the clean environment at a limited cost. Toxic heavy metals can be eliminated from the soil by specialized microbes and plants, termed as hyper accumulators, which are capable of accumulating and tolerating the toxic levels of the heavy metals in their shoots (Cunningham et al. 1995; Salt et al. 1998). However, this ability of bioremediation is limited to few organisms. A promising way of improving bioremediation potential would be to genetically engineer the organisms with enhanced abilities to tolerate and accumulate toxic heavy metals and metalloids. Though many efforts have been made to engineer the microbes and plants for an enhanced metal

accumulation potential, but limited success was achieved in this direction (Brunetti et al. 2011; Liu et al. 2012; Wang et al. 2012; Kumar et al. 2013).

The heavy metal cadmium (Cd) is one among most toxic carcinogenic elements, which show high affinity towards thiols (Tripathi et al. 2013). Cd is a widespread heavy metal that is mainly released into the environment by anthropogenic activities and it is highly toxic to both plants and animals. Cd decreases the growth rate of plants by affecting various aspects of metabolism mainly in normal uptake and utilization of macro and micronutrients (Metwally et al. 2005; Sanita di Toppiet al. 1999). Plants have developed various defense mechanisms for protection against various environmental stimuli. Exposure of plants to heavy metals, such as cadmium (Cd), arsenic (As), mercury (Hg) or lead (Ld), promotes the synthesis of plant metal chelators likes metallothioneins (MTs) and phytochelatins (PCs) (Cobbett et al. 2002). MTs are small Cys-rich proteins encoded by multigene families; while, PCs are enzymatically synthesized Cys- rich polypeptides that are likely to be ubiquitous in plants, and possibly present in animals also (Lee et al. 2002). The thiol group of Cys has been reported to play an important role in both the homeostasis of essential heavy metal ions and the sequestration of various non-essential toxic metal ions at the sub cellular level (Rauser 1995).

PCs are a family of cysteine-rich, thiol-reactive peptides that bind many toxic metals and metalloids, making them good candidates for genetically enhanced phytoremediation strategies (Cobbett and Meagher 2002). PCs were first identified in the yeast *Schizo saccharomyces pombe* and subsequently have been found in plants, fungal species, marine diatoms and certain animals (Cobbett 1999). PCs are non-translationally synthesized small polypeptides and are synthesized by the transpeptidation of γ-glutamylcysteinyl dipeptides from glutathione (GSH) with the help of Phytochelatin Synthase (PCS). PCS is a constitutively expressed enzyme and it is known for its post-translational activation in the presence of heavy metals. PCS genes have been identified in many organisms such as *Arabidopsis* (*AtPCS1*), wheat (*TaPCS1*), *S. Pombe*(*SpPCS1*) and *Caenorhabditiselegans*(*CePCS1*) (Clemens et al. 1999; Clemens et al. 2001; Sanita di Toppi et al. 2002). The genes coding for PCS have been cloned from a variety of organisms and have been over-expressed in bacteria, *Arabidopsis, Nicotianaglauca* and rice (Lee et al. 2003; Li et al. 2004; Sauge-Merle et al. 2003; Gisbert et al. 2003). Over-expression of *AtPCS1* in *Arabidopsis*, Mustard and tobacco had resulted in enhanced tolerance towards Cd (Lee et al. 2003; Li et al. 2004; Brunetti et al. 2011; Gasic and Korban. 2007; Pomponi et al. 2006; Wojas et al. 2010a & 2010b). Also, transgenic plants expressing different PCS genes such as *NnPCS1*, *TcPCS1*, *PtPCS1* and *TaPCS1*in *Arabidopsis*, tobacco and poplar resulted into accumulation of various metalloids and heavy metals likes Cd, As, Pb and Zn (Liu et al. 2012; Couselo et al. 2010; Adams et al. 2011; Liu et al. 2011).

In this paper we report the effects of over-expression of the *Arabidopsis AtPCS1* gene in rice plants. We investigated whether the over-expression is capable of increasing Cd tolerance in these transgenics. Further we also investigated the accumulation levels of Cd in the transgenic rice lines in both roots and shoots in the presence or absence of these heavy metal ions.

2. MATERIALS AND METHODS

2.1 Isolation and Cloning of Arabidopsis Thaliana Phytochelatin Synthasa

Total RNA was isolated from 15-day-old *Arabidopsis* seedlings (ecotype Columbia) using the RNeasy plant mini kit (Qiagen, Germany). Following the manufacturer's instructions, the first stand cDNA was synthesised using SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific, USA). *AtPCS1* coding sequence was amplified employing forward 5′-ATGGCTATGGCGAGTTTA-3′ and reverse 5′-CTAATAGGCAGGAGCAGCGAG-3′ primers

using *pfu* polymerase (Thermo fisher, USA). The amplified fragment was cloned at *Sma*I site of pBluescript KS (+) (Stratagene, USA), and sequenced.

2.2 Construction of pCambia 1302-hyg- CaMV35S-AtPCS1- polyA-GFP Binary Vector

AtPCS1 coding sequence was released with *Bam* HI and *Sac* I sites from recombinant pBSSK–*AtPCS1* vector and cloned between CaMV35S promoter and *polyA* terminator of pCambia1302 binary vector containing hygromycin phasphotransferase gene as plant selection marker. The resulting pCambia 1302-*hyg-AtPCS1* vector was maintained in HB101 cells and then mobilized into *A. tumefaciens* strain LBA4404 by triparental mating (Lichtenstein and Draper 1985) using the helper vector pRK2013 and the resulting binary vector was designated as *pCambia 1302-hyg- CaMV35S-AtPCS1- polyA-GFP*.

2.3. Agrobacterium-Mediated Transformation and Regeneration of Transgenic Plants

Seeds of the *indica* rice cultivar BPT5204, obtained from Indian institute of rice research (IIRR), Hyderabad, were used for genetic transformation employing *pCambia 1302-hyg- CaMV35S-AtPCS1- polyA-GFP*. Calli derived from the mature embryos were infected with the *Agrobacterium* (LBA4404) culture, and putatively transformed calli were selected on the Murashige and Skoog medium (Murashige and Skoog 1962) containing 50 to 70 mg/l hygromycin (hyg) (Raineriet al.1990). Plants regenerated from the selected hyg-tolerant calli were examined for green fluorescent protein (GFP) expression and grown to maturity in the glasshouse.

2.4. Molecular Analysis of Atpcs1 Rice Transgenic Plants

Genomic DNA was isolated from hyg tolerant and un-transformed control plants according to the method of McCouch et al (1988). PCR analysis was carried out using the three primer sets for the three expression cassettes, viz., *hyg* forward 5´-ATTTCGGCTCCAACAATGTC-3´ and *polyA* reverse, 5´-GCTCAACACATGAGCGAAAC 3´ for *hyg* expression cassette; *AtPCS1* forward, 5´-TTGGTCGTACTCAAGCTC- 3´ and *polyA*reverse 5´-GCTCAACACATGAGCGAAAC 3´ for *AtPCS1* gene and *gfp* forward, 5´- TGGAAGCGTTCAACTAGCAG-3´ and *nos* reverse 5´-TTGCGCGCTATATTTTGTTTT-3´ primers were used for *gfp* expression cassette. Southern hybridization was performed as described in Vijayan et al (2013). Genomic DNA (10 µgeach) was completely digested with *Bam*HI restriction enzyme and the restriction fragments were electrophoresed on 0.8 % agarose gel, and transferred on to Hybond-N^+ nylon membrane (GE Biosciences, Hong Kong). Following this, the cross-linking of the DNA fragments was carried out by baking in the oven at 65^{o}C. Southern and northern blot analyses were carried out using the AlkPhos Direct labelling kit and the CDP star detection reagent system according to the manufacturer's instructions (Amersham Biosciences, UK) employing *AtPCS1*and *hyg*coding sequences as probes.

2.5. Segregation Analysis of Transgenic Plants

Seeds from the primary transformants were harvested to carry out the segregation analysis. Collected T_1 seeds of rice were germinated on the selection medium containing hyg (50 mg/L). The number of sensitive and resistant seedlings was counted after 2 weeks of germination. Homozygous lines were identified by germinating T_2 resistant seeds again on selection medium containing hyg (50 mg/L).

2.6. Cadmium Tolerance Analysis of Atpcs1 Transgenic Lines

Two homozygous transgenic rice lines ($SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$) expressing *AtPCS1* of T_3 generation along with un-transformed controls were used for further Cadmium tolerance analysis. Plants were grown for 10 days on MS medium and then transferred into fresh MS media containing various concentrations of $CdCl_2$. After 10 days of treatment length of shoots as well as roots were recorded and used for Cadmium estimation.

2.7. Cadmium Estimation

Shoots and roots from hydroponic medium grown rice seedlings were collected and dried in hot air oven at 40 ºC to reach constant weight. Dried plant tissues (100 mg) were digested in HNO_3 and H_2O_2 (3:1), through microwave digestion, in Microwave digester (Roychoudhury et al. 2002). This digested solution was diluted from which 10 mL aliquot was quantitatively analysed for Cadmium through atomic absorption spectrometry (Perkin–Elmer; A Analyst 600) fitted with graphite furnace.

3. RESULTS

3.1. Cloning of Atpcs1 Into pBSSK (+)

An approximate 1.5 kb cDNA coding region of *AtPCS1* was amplified employing total RNA isolated from *Arabidopsis* seedlings. The isolated *AtPCS1* gene was ligated into *Sma*I-digested pBSSK (+) vector. The positive pBSSK (+)-*AtPCS1* clones revealed 1.5 kb fragment after BamHI and SacI restriction digestion. *At PCS1* coding sequence was cloned between CaMV35S promoter and *polyA* terminator of pCambia1302 binary vector. The positive pCambia 1302-*hyg-AtPCS1* vector revealed 1.5 kb fragment after BamHI and SacI restriction digestion. The resulting binary vector *pCambia 1302-hyg-CaMV35S-AtPCS1- polyA-GFP* was mobilized into *A. tumefaciens* strain LBA4404 by triparental mating.

3.2. Genetic Transformation and Production of Transgenic Rice Plants Using pCambia 1302-hyg-CaMV35S-AtPCS1- polyA-GFP

Transgenic rice plants of cv. Samba Mahsuri (SM) were regenerated from hygromycin resistant calli obtained after co-cultivation with the *Agrobacterium* strain LBA4404 harbouring the binary vector carrying *gfp*, *hyg* and *AtPCS1* (*pCambia 1302-hyg-CaMV35S-AtPCS1- polyA-GFP)* (Figure 1A). A total number of 12 putative transformants were obtained from 7654 calli of SM. Putative transformed calli as well as regenerated plants showed the GFP expression in all the stages studied. Whereas un-transformed calli and plants failed to express the GFP in various stages tested (Figure 1B).

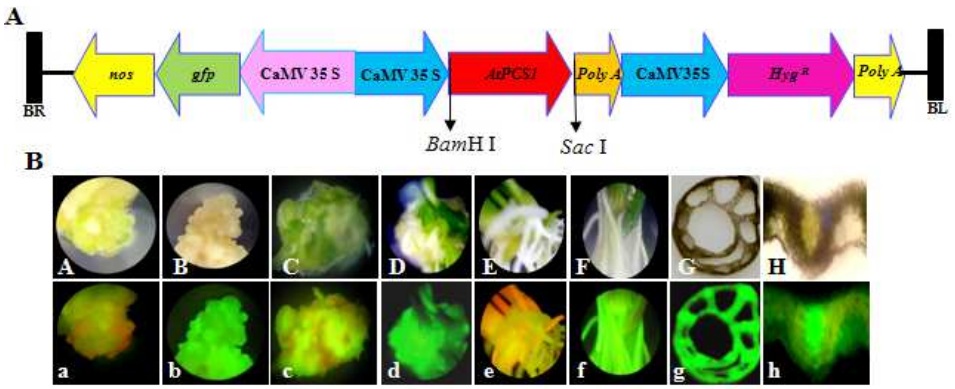

Figure 1: Restriction Map of T-DNA and GFP Expression in Untransformed Control and *AtPCS1* Transgenic Rice Plants

- Restriction map of T-DNA region of pCambia 1302 containing *hygromycin*, *gfp* and *At PCS1* expression units.
- Bright field and GFP image of control callus (A & a) transgenic callus (B & b), regenerated untransformed control calli (C & c), regenerated transformed control calli (D & d), un-transformed control shoot and root (E & e), transformed shoot and root (F & f) T.S of transformed shoot (G & g) and T.S of transformed leaf (H& h), respectively. The images were acquired with an Olympus BX65 fluorescence microscope.

3.3. Molecular Analysis of Primary (T_0) Transformants

Genomic DNA and total RNA, isolated from the GFP positive transgenic rice plants and the un-transformed control plants, were used for molecular analyses. PCR analysis of *AtPCS1*-transgenic plants, using *hyg* forward and *poly A*reverse and *AtPCS1* specific primers, showed amplifications of 600 bp and 750 bp representing *hyg* and *AtPCS1* coding sequences, respectively, while the un-transformed control plants failed to show any amplification (Data not shown). Genomic DNA of *AtPCS1*-transgenics digested with *Xho*I and probed with the *hyg* coding sequence showed a hybridizable band of ~1.1 kb (Figure 2A). Similarly, *Bam* HI digested DNA of *AtPCS1*-transformants, probed with the *AtPCS1* sequence showed different hybridizable bands of >3.6 kb corresponding to the *AtPCS1* gene (Figure 2B). Furthermore, northern blot analysis performed using the total RNA from the Southern positive plants, when probed with *AtPCS1*coding sequence, showed ~1.5 kb hybridizable band of varied intensities in different transformants of rice (Figure 2C).

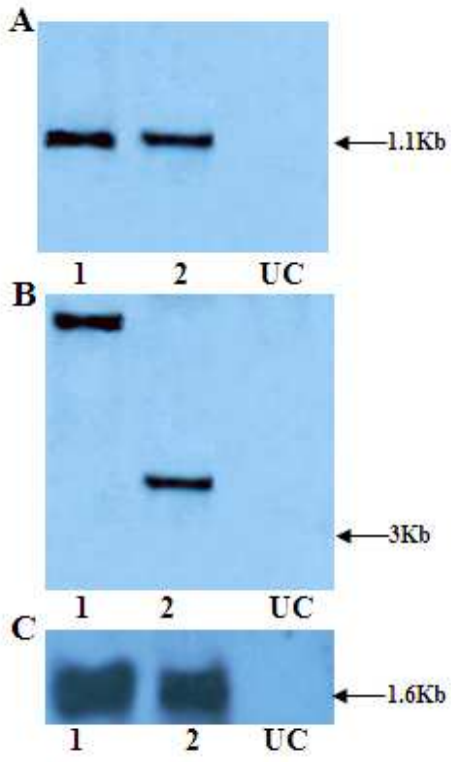

Figure 2: Southern and Northern Blot Analysis of Untransformed Control and AtPCS1 Transgenic Rice Plants

- Genomic DNA digested with *Xho* I and probed with *hyg* coding sequence

Lane UC: DNA from untransformed SM control plants; Lanes 1&2: DNA from $SMPC_2$ and $SMPC_5$ transgenic lines of SM.

- Genomic DNA digested with *Bam* HI and probed with *AtPCS1*coding sequence Lane UC: DNA from untransformed SM control plants; Lanes 1&2: DNA from $SMPC_2$ and $SMPC_5$ transgenic lines of SM.
- Total RNA probed with *AtPCS1* coding sequence. Lane UC: RNA from untransformed SM control plants; Lanes 1&2: RNA from $SMPC_2$ and $SMPC_5$ transgenic lines of SM.

3.4. Inheritance of Transgenes InT_1Generation and Identification of Homozygous Transgenic Lines Using Hygromycin (Hyg) Sensitivity Test In T_2Generation

To investigate the inheritance pattern of transgenes, selfed seed collected from the primary (T_0) transformants were germinated and T_1 progenies were grown to maturity in the glass house. Selfed seed of T_1 progenies of two SM *AtPCS1* -transgenic lines ($SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$), were germinated on MS medium containing 50 mg hygromycin. All the T_1 progenies showed distinct (3T:1S) monogenic segregations. Similarly, in T_2 generation, selfed seed of 32 T_2 progenies derived from $SMPC_{2\text{-}1\text{-}15}$and $SMPC_{5\text{-}1\text{-}10}T_1$ plants, when germinated on MS medium containing hyg, showed 6 T_2 progenies with complete germination, 18 progenies segregated into 3:1 ratio, and the seeds of 8 progenies failed to show germination. Based on progenies showing 100 % germination on hyg medium, two homozygous lines of *AtPCS1*-SM ($SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$) were selected for further studies. Moreover, homozygosity of selected *AtPCS1*-transgenic lines was validated through PCR test. All the plants of selected homozygous lines invariably exhibited 600 bp band using hygromycin specific primers (Data not shown).

3.5. Effect of Cd on Root Growth of Atpcs1 Rice Transformants and Untransformed Control (UC)

Root length was measured in homozygous rice lines expressing *AtPCS1* as well as UC against Cd stress. Initially, no significant differences were observed in root length between UC and transgenic rice lines in the absence of Cd. Significant differences were observed between the UC and transgenic rice lines on 100 µM $CdCl_2$. *AtPCS1* transformants exhibited a mean root length of 6.53 ± 0.15 in $SMPC_{2\text{-}1\text{-}15}$ compared with 5.40 ± 0.20 cm un-transformed SM (Figure 3). After Cd treatments at higher concentration roots were turned brown as the sign of heavy metal toxicity.

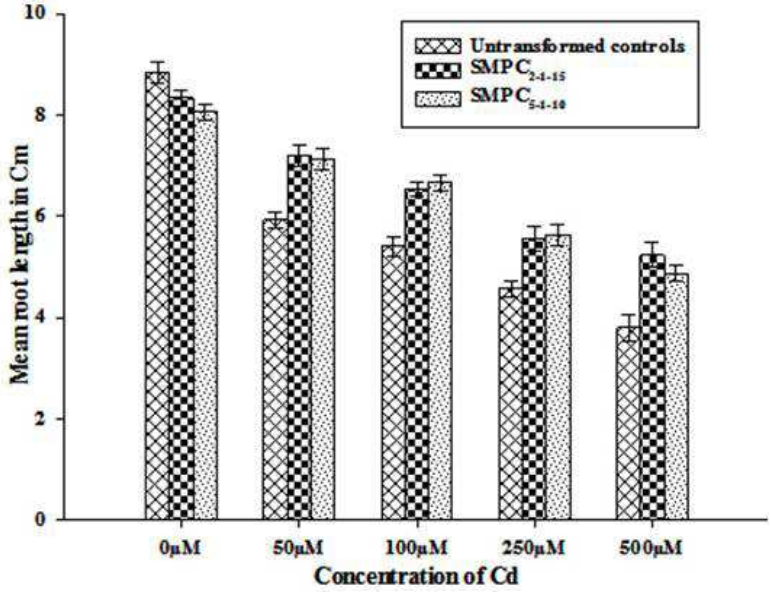

Figure 3: Effect of Cd on Root Length of AtPCS1 Expressing Lines and UC

UC and transgenic lines of rice ($SMPC_2$ and $SMPC_5$), were grown for 10 days on MS media and then transferred in fresh MS media containing various concentrations (0 µM, 50 µM,100 µM,250 µM and 500 µM) of $CdCl_2$. (A) Root length in presence of Cd was recorded after 10 days of treatment. Data are expressed as means ± SD of at least 3 independent experiments. Differences between the mean values of control vs transgenic rice lines were significant at p value <0.0001 (ANOVA).

3.6. Effect of Cd on Shoot Growth of Atpcs1 Rice Transformants and UC

Shoot length was measured in homozygous rice lines expressing *AtPCS1* and UC against Cd stress. Initially, no significant differences were observed in shoot length between UC and transgenic rice lines in the absence of Cd. Significant differences were observed between the UC and transgenic rice lines on 100 µM $CdCl_2$. *AtPCS1* transformants exhibited a mean shoot length of 14.3± 0.35 and in $SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$ compared with 16.4 ± 0.36 cm un-transformed SM (Figure 4). After Cd treatments at higher concentration phenotypically, leaves of transgenic plants turned yellow as the sign of heavy metal toxicity. Though similar effects were also noticed in UC plants, the transgenic lines were found to be more sensitive to Cd stress.

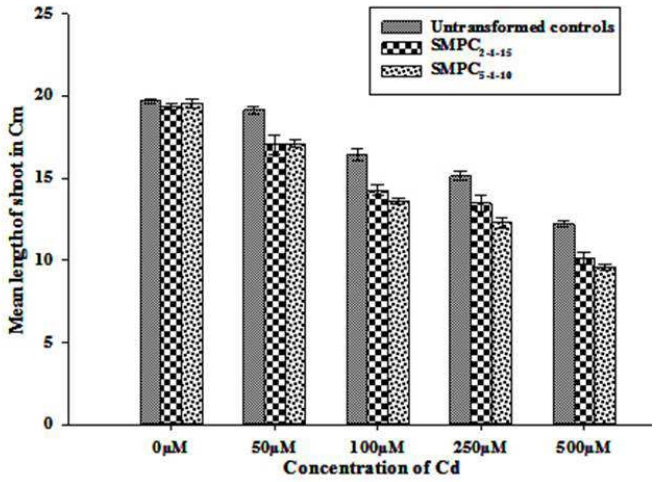

Figure 4: Effect of Cd on Shoot Length of *AtPCS1* Expressing Lines and UC

UC and transgenic lines of rice ($SMPC_2$ and $SMPC_5$), were grown for 10 days on MS media and then transferred in fresh MS media containing various concentrations (0 µM, 50 µM,100 µM,250 µM and 500 µM) of $CdCl_2$. (A) Shoot length in presence of Cd was recorded after 10 days of treatment. Data are expressed as means ± SD of at least 3 independent experiments. Differences between the mean values of control vs transgenic rice lines were significant at p value <0.0001 (ANOVA).

3.7. Cadmium Accumulation in Roots and Shoots of Atpcs1 Transgenic Rice Lines and Untransformed Controls

To analyse the partitioning of heavy metals in different parts of rice plants, accumulation of Cd was measured in roots and shoots of homozygous *AtPCS1*- transgenic rice lines and un-transformed controls. Transgenic plants accumulated significantly increased accumulation of Cd in shoots compared to un-transformed controls. Significant differences were observed between the UC and transgenic rice lines on 100 µM Cd. Transgenic plants accumulated significantly increased accumulation of Cd in roots compared to UC. Significant differences were observed between the UC and transgenic rice lines on 100 µM Cd. A mean Cd concentration of 280.36 ± 2.57 and 302.86 ± 2.3µg.g^{-1} dry weight was recorded in roots

of in $SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$, respectively, compared with 59.83 ± 4.01µg.g^{-1} dry weight of Cd in untransformed SM. Furthermore, a mean Cd concentration of 182.33 ± 7.02 and 197.33 ± 3.5µg.g^{-1} dry weight was recorded in shoots of in $SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$, respectively, compared with 72.33 ± 1.15µg.g^{-1} dry weight in untransformed SM (Figure 5A & B).

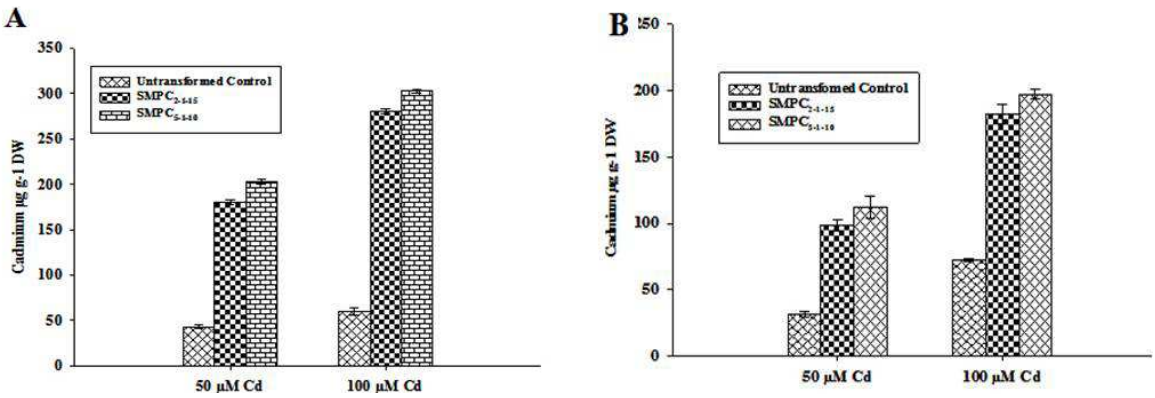

Figure 5: Cd Accumulation in Roots (A) and Shoots (B) of Untransformed Controls (UC) and *AtPCS1* Expressing Rice Seedlings

UC and transgenic lines of rice ($SMPC_2$ and $SMPC_5$) were grown for 10 days on MS media and then transferred in fresh MS media containing either 50 µM or 100 µM $CdCl_2$. After 10 days of treatment roots and shoots of UC and transgenic lines were washed, dried and accumulation of Cd was measured from 100 mg dried tissue. Data are expressed as means ± SD of at least 3 independent experiments. Differences between the mean values of control vs transgenic rice lines were significant at p value <0.0001 (ANOVA).

4. DISCUSSIONS

Phytochelatins, a class of post translationally synthesized peptides, play a key role in heavy metal, primarily Cd tolerance in plants and fungi by chelating these substances and decreasing their free concentrations. PCS is constitutively expressed and its activity mainly dependent on the presence of heavy metals (Vatamaniuk et al. 2004). This investigation deals with cloning of *Arabidopsis phytochelatin*gene (*AtPCS1*) and its effects on tolerance to Cd in transgenic rice plants.

Isolated *AtPCS1* gene and amino acid sequence showed 95-100% homology with that of other previously reported *Arabidopsis* PCS and 80-90% homology with *Brassica* species thus indicating the highly conserved nature of the enzyme. Amino acid sequence of C-terminal region of the AtPCS1 contained homologous regions to functional domains of metallothionein and thioredoxin. Therefore, it was suggested that C-terminal region of the PCS play an important role as regulatory region for PC synthesis. The arrangements of Cys residues (–Cys–Cys–Arg–Glu–Thr–Cys–Val–Lys–Cys–) is reminiscent of those found in metallothionein and thioredoxin (Cobbett et al. 2002; Capitani et al. 2000; Romero-Isart et al. 2002). The sequence homology identified pfam05023 domain which is specific to Phytochelatin synthase. This domain is responsible for the synthesis of heavy-metal-binding peptides (phytochelatins) from glutathione and related thiols. This enzyme contains a catalytic triad of cysteine, histidine and aspartate residues. Another domain DUF 1984 (Domain of unknown function) was also found in the members of this family of functionally uncharacterized domains are found at the C-terminus of plant phytochelatin synthases.

To introduce *AtPCS1* gene for developing transgenic rice tolerance to heavy metal toxicity, embryogeniccalli of elite indica rice cultivar Samba Mahsuri (SM) were co-cultivated with the LBA4404 harbouring *pCambia1302-hyg-*

CaMV35S-AtPCS1-polyA-GFP vector. The putative *ATPCS1*-SM rice transgenic lines which showed consistently higher tolerance to hygromycin in repeated tests were further subjected to molecular analyses for confirmation of integration and expression of transgenes. PCR, Southern and Northern blot analyses of hygromycin tolerant plants confirmed the stable integration of *hyg*and *AtPCS 1* genes into the *indica*rice genome. Southern blots of *Xho*I digested genomic DNA of *AtPCS1* transformants, when probed with *hygromycin*, revealed a specific hybridizable band of ~1.1 kb in each of the transformants, suggesting the stable integration of *hygromycin* cassette in the transformants of rice. Appearance of different hybridizable bands of >3.5 kb corresponding to the *AtPCS1* expression cassette with *AtPCS1* coding sequence in the *Bam HI* digested genomic DNA of these plants, indicates the single copy and independent nature of the transformants. The specific bands observed on Southern blots of transgenic plants confirm the presence of *hygromycin* and *AtPCS1* transgenes. Conversely, the un-transformed control plants failed to show specific hybridizable bands with these probes. Single copy integration of transgene(s) is essential to achieve predictable patterns of inheritance and to eliminate the problem of gene silencing in transgenic plants (Finnegan and Mc Elroy 1994).

Furthermore, northern blot analysis showed variable expression of *AtPCS1* gene in the primary transgenic plants as evidenced by varied intensity of the hybridizable bands of >1.5 kb when *ATPCS1* gene employed as probe. The level of transgene expression was variable and influenced by many factors, such as, integration site, transgene copy number, transgene locus conFigureuration, epigenetic silencing factors, and flanking matrix attachment regions (Kumpatla and Hall 1998; Morinoet al. 2004; Yang et al. 2005). It is well established that the transgenes present in multiple copies as well as transgenes that are related to homologous endogenous sequences invariably show gene inactivation (Finnegan and McElroy 1994).

To establish the definitive transgenic nature of primary transformants, the inheritance pattern of transgenes was analysed in the T_1 and T_2 generations. Segregation analyses of transgenes in T_1 progenies revealed a monogenic ratio of 3 resistant: 1 tolerant plant(s) for hygromycin tolerance, suggesting that these genes are stably integrated into the rice genome. The co-segregation of transgenes affirms that both *hyg-AtPCS* are integrated and manifest as a single locus. Homozygous transgenic rice lines, expressing *AtPCS1* gene, were identified in T_2 generation, employing hygromycin sensitivity test. From these, two homozygous lines of *AtPCS*1- ($SMPC_{2\text{-}1\text{-}15}$ and $SMPC_{5\text{-}1\text{-}10}$), transformants were selected for further evaluations.

Tolerance to Cd was evaluated in transgenic and un-transformed control plants. The shoot growth in terms of length was affected more in transgenic lines when exposed to Cd. Cd produced a significant decrease in shoot length compared with the control. Shoot length was gradually reduced by 15-50 % in the Cd treatment of 50-500 µM respectively. Also, root length was decreased by 24-96 % in the Cd treatments of 50- 500 µM, respectively. The effect was more prominent in root compared to shoot because these are the primary site of defence. There was no significant difference in root length of the transgenic rice lines and un-transformed controls in the absence of Cd. Even at 50 µM of Cd decrease in root length was significant in transgenic rice lines but this decrease was more at 100 µM Cd. In presence of 100 µM Cd there was 20-25 % reduction in shoot and root length of transformants to un-transformed controls.

Earlier, it was reported that, expressing PCS genes from *Arabidopsis* (*AtPCS1*) and wheat (*TaPCS1*) in rice did not confer tolerance to Cd instead Cd sensitivity (Wang et al., 2012; Lee et al, 2003; Li et al, 2004). Transgenic *Arabidopsis* plants over-expressing a native *AtPCS1* exhibited Cd hypersensitivity (Lee et al. 2003; Li et al. 2004), while transgenic tobacco plants expressing a wheat *TaPCS1* showed slight increases in Cd tolerance (Gisbert et al. 2003). The

observed discrepancies were attributed to possible functional differences between *AtPCS1* and *TaPCS1* enzymes, and more likely attributed to differences in downstream processing of Cd-PC complexes between tobacco and *Arabidopsis* (Li et al. 2004) as well as to the toxicity of phytochelatins at supra-optimal levels (Lee et al. 2003).

Cd content was measured in un-transformed controls and homozygous *AtPCS1* transgenic lines grown under Cd exposure of 50 and 100 µM for 10 days. All the transgenic lines accumulated significantly higher amount of Cd. Transgenic lines accumulated more Cd in roots as well as shoots in comparison to UC. Roots accumulated more Cd in comparison to shoots.

The overall results clearly suggest that *AtPCS1*-expressing transgenic rice lines exhibit higher-levels of Cd accumulation in both roots and shoots when compared to UC. It was reported that heterologous expression of *CdPCS1* in tobacco and *Arabidopsis* led to significantly enhanced Cd accumulation in roots accompanied by increased PCs content (Shukla et al. 2012 & 2013). Furthermore, ectopic expression of *CdPCS1* complements cad1-3 (PC-deficient) mutant of *Arabidopsis* to the similar level of synthetic PC (Shukla et al. 2013). Earlier, it was reported that expression of *NnPCS1* in *Arabidopsis*, *PtPCS1* and *TaPCS1* in poplar and *TcPCS1* in tobacco disclosed accumulation of various metalloids and heavy metals, such as Cd, As, Pb and Zn (Liu et al. 2012; Couselo et al. 2010; Adams et al. 2011; Liu et al. 2011). Whereas, heterologous expression of *TaPCS1* in rice, over-expression of *AtPCS1* in *Arabidopsis* resulted in Cd-hypersensitivity (Wang et al. 2012; Lee et al 2003; Li et al 2004). These disparities in transgenic plants expressing PCS might have arisen due to differential PCS activity in source genes and nature of plant species selected for transformation. There are enough evidences which suggest the role of PCs in the long-distance transport of heavy metal (loid)s via either xylem or phloem (Lappartient and Touraine 1996; Li et al. 2006; Gong et al. 2003; Chen et al. 2006; Mendoza et al. 2008). A similar kind of mechanism might be involved in *AtPCS1*-expressing rice plants which has led to higher heavy metal(loid)s accumulation in both roots and shoots.

5. CONCLUSIONS

This investigation deals with the cloning and expression of *Arabidopsis phytochelatin*gene (*AtPCS1*) in transgenic rice plants. The transgenic rice lines showed marked enhancement in tolerance to Cd and also disclosed increased accumulation of these heavy metals both in roots and shoots.

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